Semi-Aquatic
Sea Snakes

Aipysurus +
Emydocephalus

Notechis

Terrestrial
Australian
Elapids

Laticauda

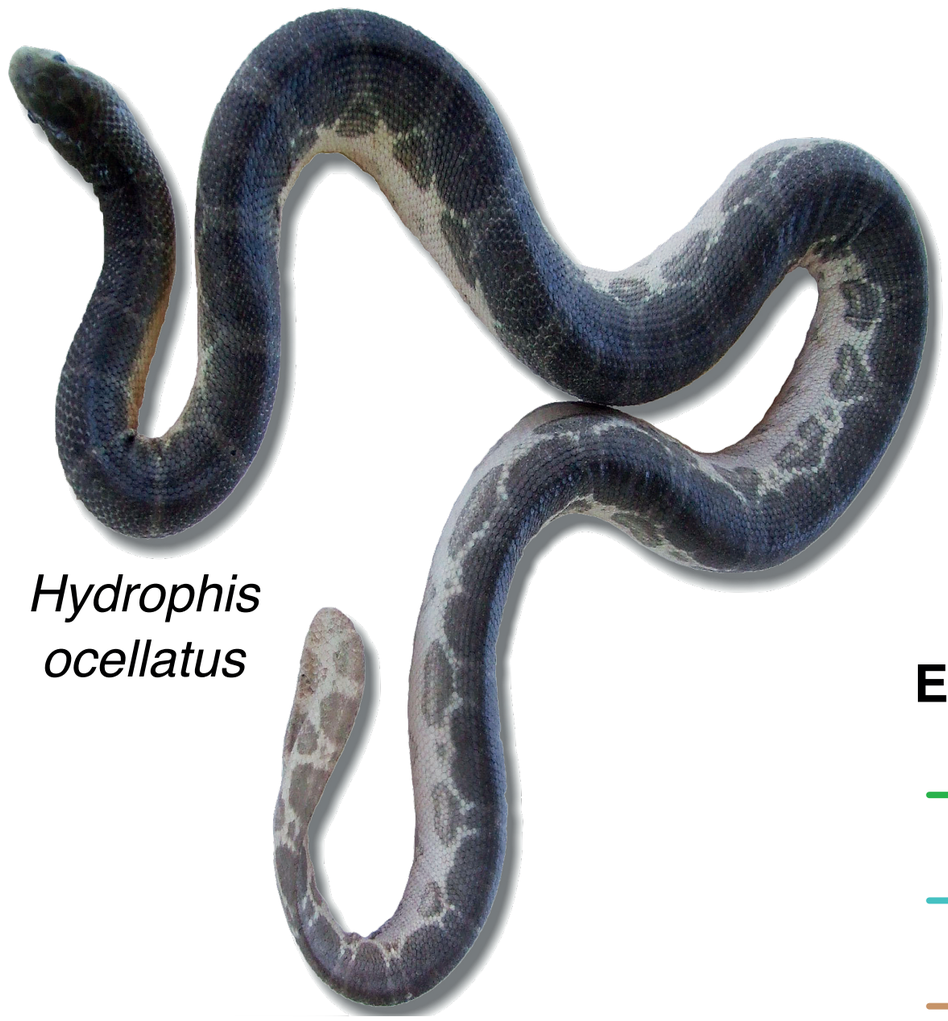
Other Elapid
Snakes

Hydrophis

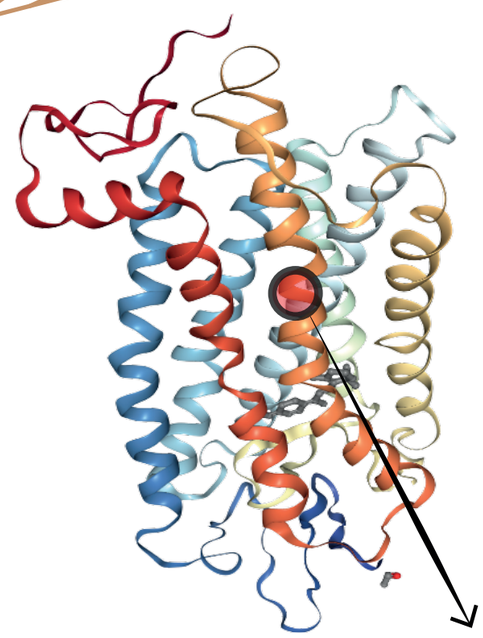
Ancestral
Trans-Species
Polymorphism

Sea Kraits

Sea Snakes



Hydrophis ocellatus



Short-Wavelength Opsin 1 (SWS1)

SWS1 λ_{max}

C86

F86 Y86 S86

UV (~360nm) Visible (<400nm)

Ecology

Marine

Semi-Aquatic

Terrestrial

Current Biology, in press

Spectral diversification and trans-species allelic polymorphism during the land-to-sea transition in snakes

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Summary

Snakes are descended from highly visual lizards [1] , but have limited (probably dichromatic) colour vision attributed to a dim-light lifestyle of early snake ancestors [2–4]. The living species of front-fanged elapids, however, are ecologically very diverse, with ~300 terrestrial species (cobras, taipans, etc.) and ~60 fully marine sea snakes, plus eight independently marine, amphibious sea kraits [1]. Here, we investigate the evolution of spectral sensitivity in elapids by analyzing their opsin genes (which are responsible for sensitivity to UV and visible light), retinal photoreceptors, and ocular lenses. We found that sea snakes underwent rapid adaptive diversification of their visual pigments when compared with their terrestrial and amphibious relatives. The three opsins present in snakes (SWS1, LWS, RH1) have evolved under positive selection in elapids, and in sea snakes have undergone multiple shifts in spectral sensitivity towards the longer wavelengths that dominate below the sea surface. Several distantly related *Hydrophis* sea snakes are polymorphic for shortwave sensitive visual pigment encoded by alleles of SWS1. This spectral site polymorphism is expected to confer expanded 'UV-Blue' spectral sensitivity and is estimated to have persisted twice as long as the predicted survival time for selectively neutral nuclear alleles. We suggest that this polymorphism is adaptively maintained across *Hydrophis* species via balancing selection, similarly to the LWS polymorphism that confers allelic trichromacy in some primates. Diving sea snakes thus appear to share parallel mechanisms of color vision diversification with fruit-eating primates.

Key words: vision, evolution, snakes, trans-species spectral polymorphism, balancing selection

Results

Visual pigments detect light and underpin colour vision by differential absorption across the electromagnetic spectrum. The spectral location of the wavelength of peak absorbance (λ_{max}) of visual pigments is strongly correlated with substitutions at 'spectral tuning' amino acid sites within or close to their opsins' retinal binding pockets. We examined

the spectral characteristics of the three opsins present in elapids (Long-Wavelength Sensitive - LWS, Short-Wavelength Sensitive type 1- SWS1 and Rhodopsin type 1 - RH1) by sequencing these genes for 29 terrestrial and marine species and generating 149 additional sequences of SWS1 exon 1 for 48 (of the 62 recognized) sea snake species. Inferences of spectral changes based on substitutions at spectral tuning sites (λ_{max}, Figures 1 and S1) closely matched measurements of photoreceptor spectral absorbances determined from microspectrophotometry (MSP) for one terrestrial and five fully marine species (Figures 2 and S2). Most elapid snakes may use all available light for vision given new data showing highly transmissive lenses in all sampled species other than *H. stokesii* which appears to have lenses that partially filter UV wavelengths (Figure S3A).

SWS1 opsins varied primarily at spectral site 86, one of the sites thought to underlie spectral tuning in SWS1 opsins [2,3,5]. A phenylalanine was found at site 86 (F86) in all terrestrial elapids and the amphibious sea krait *Laticauda colubrina* and is the inferred ancestral condition for caenophidian (and colubroid) snakes [4]. Previous λ_{max} estimates obtained using MSP [4,6,7] suggest that F86 is associated with UV sensitivity in all these species. This is further supported by our SWS1 pigment MSP data for the terrestrial *Notechis scutatus*, with a mean λ_{max} value of ~366nm (Figure S2). For all 34 individuals sequenced of the nine fully marine *Aipysurus-Emydocephalus* species sequenced we recovered serine at site 86 (S86), indicating a phenylalanine to serine substitution (F86S) in the common ancestor of this clade, and implying a shift in SWS1 visual pigment sensitivity away from UV towards sensitivity in the 'blue' region of the visible spectrum. In elephants, the wildtype S86 gives a λ_{max} at 419nm, with site-directed mutagenesis S86F resulting in an ultraviolet pigment [8,9]. The semiaquatic, monotypic sister lineages to *Hydrophis* (*Ephalophis* and *Parahydrophis*) have cystaine at site 86 (C86), which is found outside squamates in lemurs and the common cormorant where SWS1 pigment λ_{max} values are >400nm [10,11] implying that this amino acid also results in a shift in sensitivity from UV to longer wavelengths, although site-directed mutagenesis studies in birds have yielded conflicting results with respect to C86 [11].

The UV-sensitivity related F86 amino acid site was observed in the SWS1 of the two sister lineages to the *Hydrophis* radiation: the semiaquatic *Hydrelaps darwiniensis* and fully marine *Microcephalophis gracilis*. Within *Hydrophis*, we detected two SWS1 variants, one displaying the F86 present in its aquatic sister lineages (and in terrestrial snakes), and one with a tyrosine at spectral site 86 (Y86). MSP data (Figure S2) for the Y86 homozygous (Table S2) *Hydrophis stokesii* suggests that this SWS1 variant is longwave-shifted (mean λ_{max} ~428nm). Additionally, MSP of *Hydrophis curtus* and *H. peronii* yielded mean SWS1 pigment λ_{max} values of 428 and 430nm, respectively [12], for photoreceptors characterized by the Y86-SWS1 opsin variant. We thus infer that the F86Y substitution longwave-shifts the sea snake SWS1 pigments from UV-sensitive (UVS) to Visible-sensitive (VS).

Unexpectedly, at least six *Hydrophis* species are polymorphic at site 86, having both F86 and Y86 alleles (Figure 1). *H. cyanocinctus* and *H. atriceps* included both heterozygous (FY86) and homozygous (F86 or Y86) individuals. Both F86 and Y86 homozygotes occur in populations of *H. fasciatus* (sister to *H. atriceps*), and in three species closely related to *H. cyanocinctus* (*H. melanocephalus, H. parviceps, H. spiralis*) (Figure 1, Table S3). We suggest that these species are also likely to be polymorphic in their SWS1 because F86 and Y86 homozygotes shared many of the same (or similar) mitochondrial haplotypes (not shown, but see [13,14]) and were sampled from the same locations. Thus F86/Y86 SWS1 polymorphic species appear to occur in two clades (the *H. cyanocinctus* group *sensu lato* and the *H. fasciatus-atriceps* complex) that are distantly related within the *Hydrophis* phylogeny (Figure 1).

Allelic states (polymorphic or fixed for F86 or Y86 in SWS1) are still unclear for at least 25 *Hydrophis* species for which few individuals were sampled. Nevertheless, some more densely sampled lineages appear to be fixed for either allele, including *H. caerulescens* (all 8 individuals F86 homozygotes), *H. curtus* (all 10 individuals Y86 homozygotes), and possibly also the wider *H. ornatus* clade based on 20 individuals (across eight species) that were all Y86 homozygotes. It appears that although Y86 evolved relatively early in *Hydrophis* and was independently fixed in some taxa, it persisted via an ancestral multispecies polymorphism (Figure 4A). The theoretical time to fixation for a selectively neutral nuclear allele

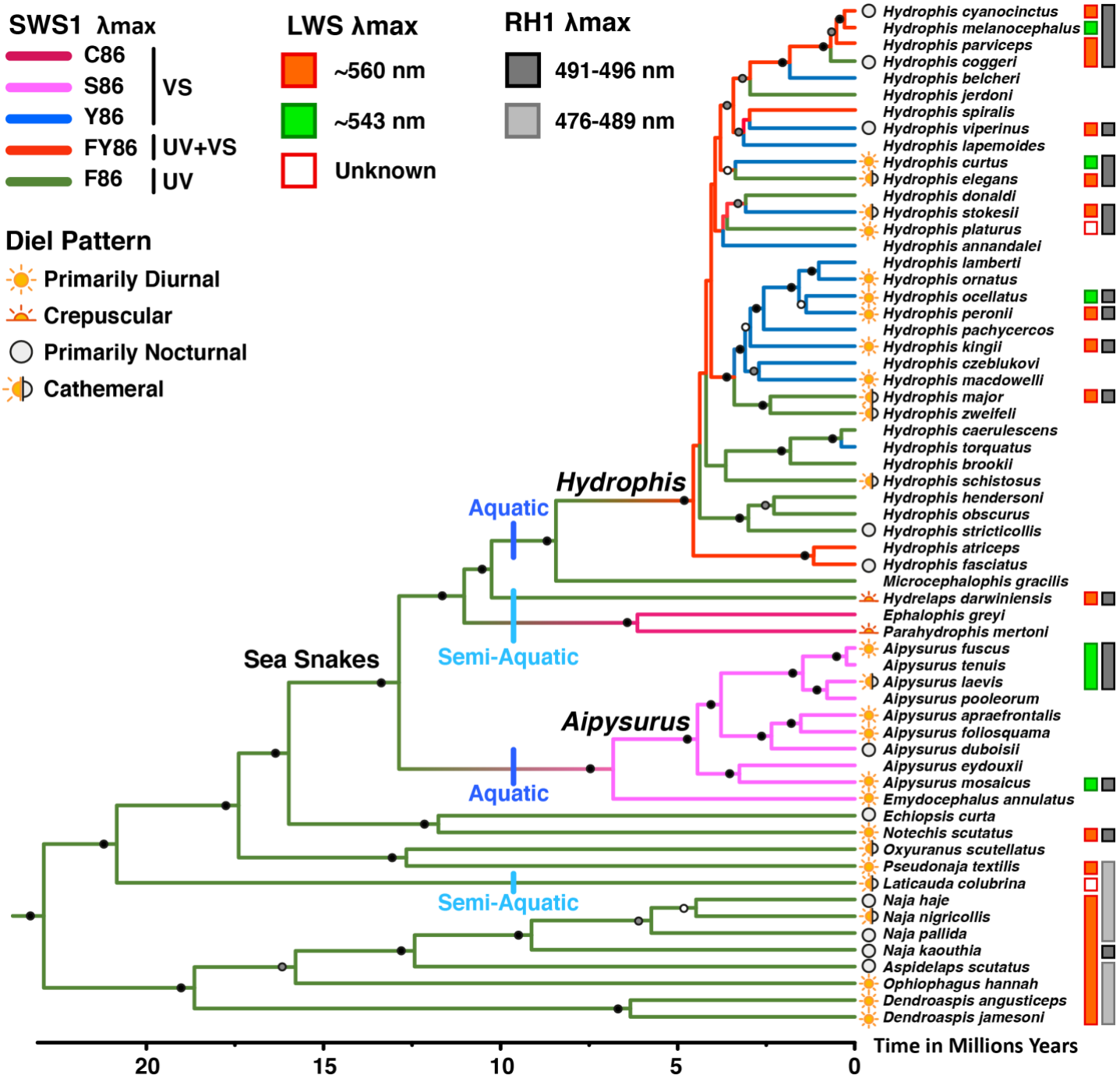

Figure 1. ***Opsin peak wavelength sensitivities predicted from DNA sequences in elapid snakes.*** *BEAST maximum credibility tree for elapids. Lineages are terrestrial unless otherwise noted. At each internal branch we show Bayesian posterior probabilities (BPP): black BPP = 1; grey = <0.9–1; white = <0.5–0.9. Visual pigment peak absorbance (λ_{max}) values for each visual pigment are those predicted from cDNA sequences (Table S2) and from MSP data (Figure S2). UV, ultraviolet sensitive (λ_{max} c. 360nm) and VS, visible spectra sensitive (λ_{max} >410nm). Diel patterns based on the literature and our analysis (Figure S3B) are shown for some species.*

is approximately 447,000 generations based on the mean effective population size of snakes (Ne of 111,771; 23,740 – 163,205)[15]. Given a clade age for *Hydrophis* of five million years and a generation time of 5 years, we estimate that the FY86 polymorphism has been maintained for ca. 1 million generations, but note that this is a conservative estimate given

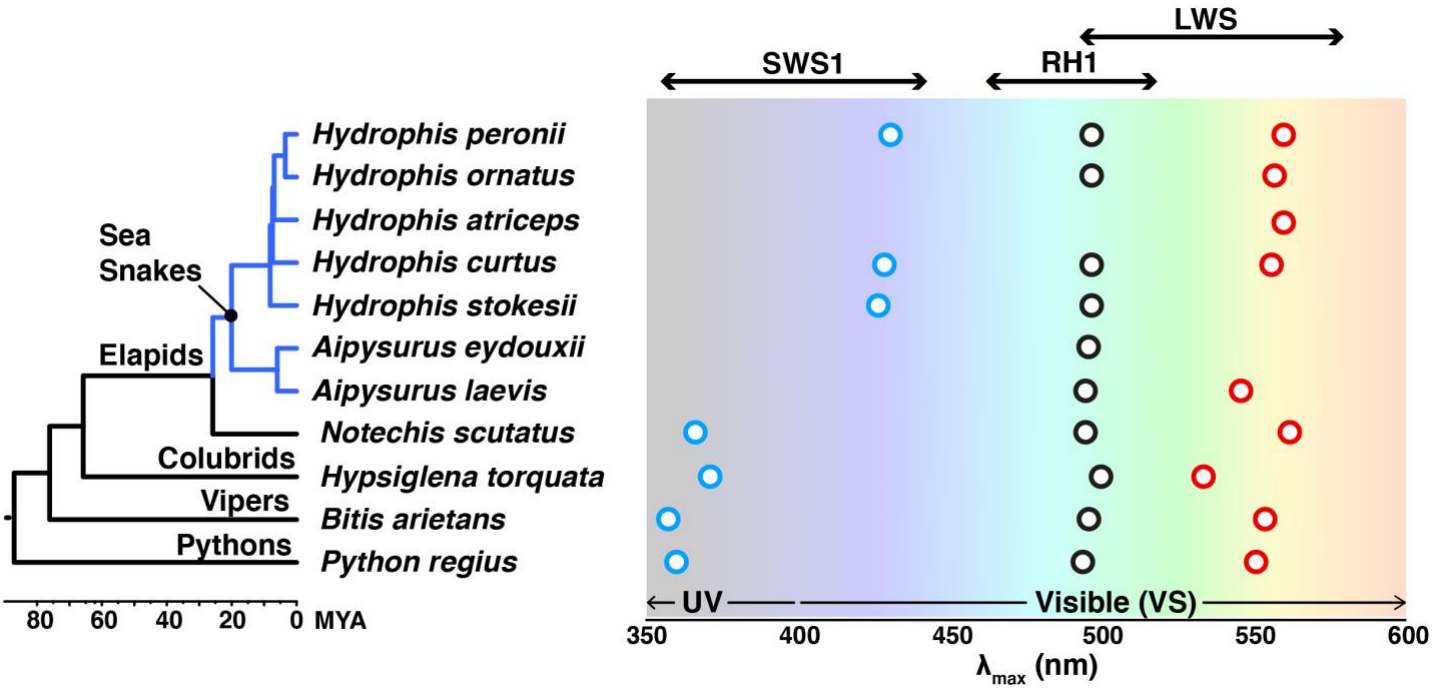

Figure 2. ***Peak wavelength sensitivities (λ_{max}) associated with each opsin-based visual pigment in snakes obtained through microspectrophotometry***. *λ_{max} from MSP for H. curtus and H. peronii were obtained from [12] and for vipers, colubrids and pythons were obtained from [41–43] (see also Figure S2)*

that our age for *Hydrophis* is a minimum date based on fossil data. Hence, the FY86 polymorphism has persisted for at least twice as long as the expected survival time for a neutral nuclear allele. Additional evidence for trans-species evolution of polymorphic alleles can be found if substitutions in the vicinity of the selected site are shared across species. However, inspection of the ~210bp SWS1 exon 1 alignment for *Hydrophis* reveals few variable sites with which to test hypotheses of recurrent evolution versus retention of polymorphism (Table S2).

Most terrestrial elapids and the amphibious sea krait opsins have RH1 (rhodopsin) pigment λ_{max} values in the range 476–489 nm [16–18], similar to the last common colubroid ancestor [4]. Sea snakes and their terrestrial sister lineage, *Notechis scutatus* (tiger snakes), have a substitution at tuning site 296 (S296A), which is inferred to long-wavelength shift the RH1 λ_{max} based on MSP measurements for *N. scutatus* and the sea snakes *A. laevis* and *H. stokesii* (λ_{max} values 494–496nm). LWS pigments have λ_{max} estimates of ~560 nm in all terrestrial and ten fully marine elapids; this is based on their spectral sites [18] and our estimated λ_{max} 556–560nm from MSP in *Notechis* and *Hydrophis* (Figures 2 and S2). At LWS tuning site 285, a T285A substitution was detected in all four *Aipysurus* species sequenced and in three *Hydrophis* that are not closest relatives. This indicates at least four independent green-shifts based on our MSP measurement of 545nm for *Aipysurus laevis* (Figures 2 and S2). Other variable LWS tuning sites exhibited substitutions only in *L. colubrina* and/or *H. platurus* (Table S4), but we were unable to make λ_{max} predictions for these combinations because they have not been reported in other vertebrates.

For all three genes, branch models suggest higher nonsynonymous/synonymous (dN/dS or ω) ratios [19] in each of the major sea snake lineages (Table S3). Clade Model C [20], for wich an extra ω is estimated among ecological partitions in the elapid phylogeny was implemented to test whether long term shifts in selective constraints were coincident with the transition to aquatic or semi-aquatic environments (Table S2). This model is a better fit to the data than the null hypothesis (M2a_rel, [21]) which does not permit a different ω to be estimated along the elapid phylogeny. Site models suggest a higher number of SWS1 amino acid sites with evidence of positive selection in the extracellular and cytoplasmatic loops than in transmembrane domains; and positive selection at 10 sites located in the functionally

important [22] transmembrane domains of both RH1 and LWS opsin (Figure 2, Table S3). Spectral tuning sites under positive selection include the SWS1 site 86 (responsible for UV to VS transitions and polymorphic across *Hydrophis*), RH1 site 292 (responsible for the long-wavelength shift in sea snakes and tiger snakes), and LWS site 285 (responsible for the green shift in *Aipysurus* and some *Hydrophis*) (Figure 3).

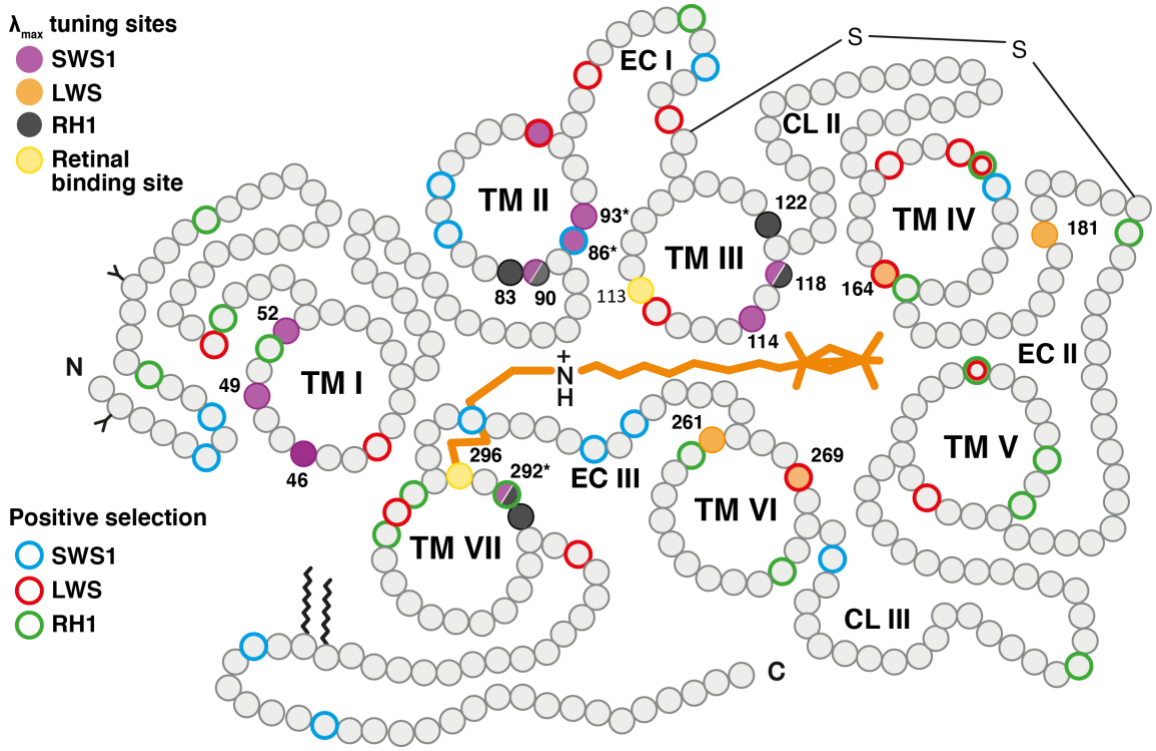

Figure 3. ***Positive selection at opsin amino acid sites.*** *Two-dimensional diagram illustrating the arrangements of the seven transmembrane (TM) domains in visual opsins around the retinal chromophore. Numbering of amino acid sites is based on bovine rhodopsin. Sites known to impact spectral tuning in vertebrate visual pigments are shown for each of the three visual pigments found in snakes. Also indicated are sites inferred to be under positive selection estimated by Bayes Empirical Bayes (model M8 ϐ&ω, see also Table S3). EL and CL are extra- and intracellular loops, respectively.*

Discussion

Our study provides the first evidence of where, when, and how frequently adaptive shifts in colour vision occurred in the ecologically diverse elapid snake radiation. Sea snakes are shown to have undergone accelerated diversification of their visual pigments, coincident with changes in photic conditions experienced during their marine invasion. Clear ocean water selectively absorbs and scatters short (UV to violet) and long (yellow to infra-red) wavelengths, causing the available light spectrum to become narrower and dimmer with increasing depth [23]. Most sea snakes forage in benthic habitats at depths of 5–100m. The longwave shifts of their pigments towards the visible violet-blue and green/yellow parts of the spectrum thus conform to expectations for spectral matching of visual pigments, whereby peak sensitivity corresponds to locally available light spectra. Interestingly, visible-spectrum shifted SWS1 pigments must have evolved independently in the major sea snake lineages, because each of these lineages has undergone a different spectral site change (to F86, S86 and C86 in *Hydrophis*, *Aipysurus-Emydocephalus* and *Ephalophis-Parahydrophis*, respectively) (Figure 1).

There remains a great deal to be learned about selection pressures on the visual sensitivities of sea snakes. The >60 extant species occupy a broad range of photic habitats and exhibit diverse behaviors [24] (Figure S3). Repeated changes

to longwave-shifted SWS1 and/or shortwave-shifted LWS pigments (Figure 1) occur in species that variously inhabit shallow and deep waters, and feed on fish eggs or hunt fish in crevices/burrows. At least nine species are strongly diurnal as well as having visible-spectrum shifted SWS1 pigments, and two of these also have green-shifted LWS pigments, indicating selection for further VS shifted vision (Figure 1, 2 and S3B). Yet most other sampled species appear to have activity patterns that depend on tidal patterns, thus are active during day and night, and have variably VS or UV sensitive SWS1 pigments. We suggest that selection pressures in addition to diel activity patterns must influence spectral sensitivities in sea snakes.

Origin and maintenance of SWS1 polymorphism in sea snakes. The presence of a F/Y polymorphism at SWS1 site 86 of several *Hydrophis* species was unexpected. Spectral site polymorphisms have been reported in only a few vertebrates and are well-studied only in the LWS opsin gene in some primates [16,25,26]. Although gene duplication cannot be ruled out, the most likely explanation is that the polymorphism arises from allelic variation within a single SWS1 copy, as seen in primates where polymorphism of the X chromosome-linked LWS opsin gene confers trichromacy in heterozygous females [16,25,26].

At the species level, recurrent changes at opsin tuning sites are usually interpreted as convergent mutations. If the Y86 mutation has convergent origins in *Hydrophis* sea snakes, it must have evolved recurrently in six or seven lineages within the last ~5 million years. Such rapid evolution is not unprecedented at spectral tuning sites (e.g. in cichlid fish [27–29]). It is possible that polymorphic *Hydrophis* lineages independently acquired the Y86 mutation, but too recently for it to have reached fixation. However, an alternative explanation is also plausible given the recent origin of the *Hydrophis* clade, high levels of species' range overlap, and the observation of FY86 polymorphism in species from two clades that are distantly positioned in *Hydrophis* phylogeny. In this scenario, the Y86 allele may have evolved early in the *Hydrophis* radiation and been maintained (or shared) across species, with some species then becoming fixed for either F86 or Y86 and others retaining both alleles (Figure 4).

The lack of variable sites in our alignment of 115 SWS1 exon 1 sequences negates phylogenetic inference of recurrent evolution versus retention of Y86 alleles within the *Hydrophis* species tree. However, our results show that the FY86 polymorphism has persisted much longer than the expected survival time for neutral nuclear alleles. This provides evidence that the Y86 allele had an ancestral origin within *Hydrophis*, and was transmitted among species, either vertically (from ancestor to descendent species) or horizontally (via interspecific introgression). Our estimates based on snake population sizes suggest that the fixation rate is ~45% of our estimate of 1 million generations for the duration of the FY86 polymorphism. Additionally, the absorption time [30] for a shared polymorphism in *Hydrophis* is ~2.7M generations, i.e. 2.7x above the approximate *Hydrophis* generation number.

Trans-species polymorphisms such as we infer for SWS1 alleles in sea snakes are often explained by dynamic, balancing selection pressures. In platyrrhine [25] and prosimian [30,31] primates, balancing selection is thought to adaptively maintain spectral tuning site polymorphisms in visual opsins across species due to heterozygous advantage favoring trichromatic females [33]. If sea snakes with SWS1 FY86 genotypes are also trichromatic (which would require the UV F86 and VS Y86 alleles to be separately expressed in photoreceptors in the retina) or have increased short-wavelength light sensitivity due to co-expression of both alleles, similar balancing selection pressures might maintain polymorphism in the *H. cyanocinctus* and *H. atriceps-fasciatus* clades. These snakes forage during dim-light periods in benthic habitats at depths exceeding 80m, yet must swim to the surface to breathe at least once every few hours throughout the diel cycle. Hence, in the absence of a large repertoire of cone opsins, allelic polymorphisms might provide a mechanism for sea snakes to both broaden their spectral sensitivities and enhance color vision in highly varied photic conditions. It is notable that the only other example of opsin polymorphism in reptiles is also found at SWS1 site 86 in an aquatic snake; several species in the colubrid *Helicops* actively forage in aquatic habitats and have a SWS1 FV86

polymorphism [4,34]. Further studies are needed to examine the mechanism and functional significance of tuning site polymorphisms in *Hydrophis* and *Helicops*, particularly to determine whether one or both spectral alleles are expressed per photoreceptor cell.

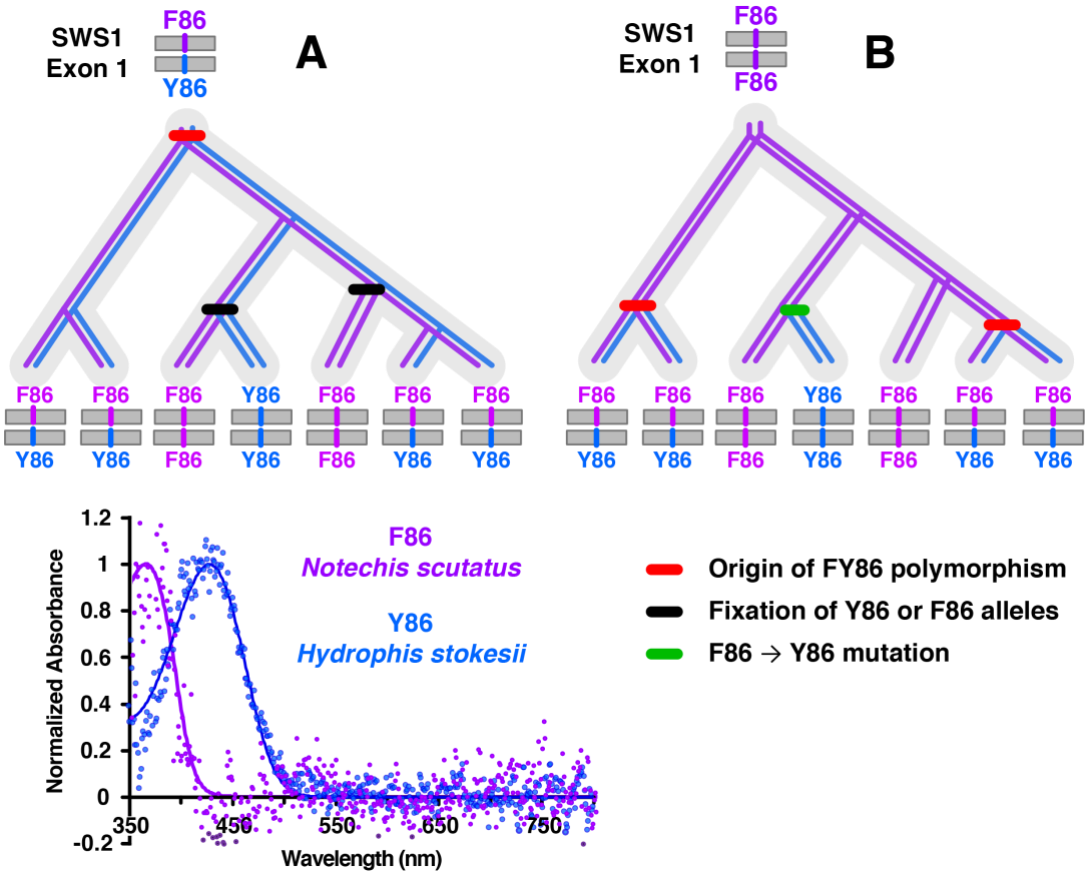

Figure 4. ***Illustration of two alternative hypotheses for the evolution of polymorphism in sea snake UV and visible-spectrum sensitive alleles (UV/VS, based on FY86).*** *Hypothesis A: The FY86 polymorphism arose early in the Hydrophis radiation, with some lineages fixing either the UV sensitive allele (F86) or the VS allele (Y86), and other lineages maintaining the FY86 polymorphism via balancing selection pressures. Hypothesis B: The ancestor of all Hydrophis sea snakes was UV sensitive (similar to terrestrial elapids and the ancestral colubroid snake [4]) with a F86 SWS1, and multiple lineages independently acquired a F86Y mutation. Some Hydrophis transitioned between UV and VS by fixing the Y86 allele, and others retained both the UV and VS alleles (the FY86 polymorphism) (see also Figure 1). Microspectrophotometry for the SWS1 for elapid species with a F86 (Notechis scutatus) and Y86 (Hydrophis stokesii) is also shown (see also Figure S2).*

Comparison with the visual systems of other secondarily aquatic vertebrates. Snakes and placental mammals share similar patterns of loss and retention of visual opsins following a dim-light bottleneck in their ancestors [17,35], and both groups contain lineages that secondarily invaded the marine environment. However, our study shows that snake and mammal visual systems have undergone fundamentally different evolutionary responses to the land-sea transition: sea snakes show adaptive diversification of color vision compared to their terrestrial relatives, whereas pinnipeds and cetaceans underwent a further reduction in the number of cone opsins, and concomitant reduction in the dimensions of their color vision [36–39]. Loss of cone opsin genes is also observed in secondarily aquatic diving birds such as penguins, grebes and pelicans, and even in non-diving aquatic foragers such as flamingos [40]. This contrast between sea snakes and other secondarily aquatic vertebrates is further evidence of the remarkable evolutionary diversity of snake visual systems.

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Authors Contribution: BFS, DJG and KLS conceived the study. BFS, DJG and KLS conducted fieldwork. ARR, MARS, NRC and RAH provided samples. BFS and KLS did the laboratory work and assembled the opsin genes. Phylogenetic and comparative genomic analyses were performed by BFS with input from BSWC, DP and KLS. MSP was done by NSH and JCP. Lens spectrophotometry was done by BFS. Trawl data were collected by GCF and analyzed by KLS. Results were interpreted by BFS and KLS with input of JCP, NSH, DMH, DJG, DP and BSWC. The manuscript was written by BFS, DP and KLS with input from all coauthors. All authors approved the final version of the manuscript.

Declaration of Interests: We have no competing interests.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Bruno F. Simões: buno.simoes@me.com

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

All opsin gene sequences generated in this study have been deposited to GenBank (MT337744 - MT337990, accession codes in Table S1). The other datasets generated during the current study are available from the corresponding author on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

This study used DNA tissues and eye samples from sea snakes (Elapidae: Hydrophiinae) and other terrestrial elapids (Elapidae) that were collected by the authors and their collaborators during fieldwork (under animal ethics compliance and scientific research permits) or loaned from museum collections.

METHOD DETAILS

Experimental Design and Taxon Sampling

Snakes were acquired through fieldwork, museum collections, Venom Supplies Ltd Pty and the Liverpool School of Tropical Medicine (Table S1). Snakes were caught, handled and euthanized using approved procedures (Liverpool School of Tropical Medicine Animal Welfare; Ethical Review Board and the UK Home Office, Schedule 1; University of Adelaide Animal Ethics Committee approval (S-2015-119); University of Western Australia AEC approval RA/3/100/1030; University of Queensland AEC approval SBS/323/08/ARC). Permits for the collection and export of sea snakes were granted by the Western Australia Government (Department of Parks and Wildlife SF010002, SF009540) and the Queensland Parks and Wildlife Service (WISP04953508; WISP05017908). Our sampling for RNA extraction aimed to cover most ecological (terrestrial, semiaquatic, costal and fully marine) and phylogenetic diversity across elapid snakes (Elapidae) but was limited by the use of approved procedures, logistic limitations with working with venomous snakes, and consideration for the impact on local populations. After euthanasia eyes were extracted, lenses removed and eyes coarsely macerated and stored in RNAlater at -80ºC until RNA extraction. Opsin genes for *Aipysurus tenuis*, *A. laevis*, *A. mosaicus*, *A. tenuis*, *Hydrophis coggeri*, *H. curtus*, *H. cyanocinctus*, *H. elegans*, *H. kingii*, *H. major*, *H. melanocephalus*, *H. parviceps*, *H. peronii*, *H. viperinus, Hydrelaps darwinensis, Laticauda colubrina, Notechis scutatus* and *Pseudonaja textilis* have been mined from genomes and eye transcriptomes. Polymorphism was checked via cloning of the opsin genes amplified directly from cDNA and via close inspection of chromatograms from Sanger sequencing. Both cloning and Sanger sequencing provide greater confidence for identification of polymorphisms than can be obtained from transcriptomes. For amplification of exon 1 of the SWS1 opsin gene we covered 48 of 62 (77%) species of sea snakes currently known. In the sea snakes (Hydrophiinae) we amplified SWS1 exon 1 in between 1 and 13 specimens per species based on sample availability, collected during more than two decades of field expeditions.

Molecular Procedures

Total RNA was extracted from eyes using TRIzol followed by purification with PureLinkTM RNA Mini Kit using the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized with a Transcriptor First Strand cDNA Synthesis Kit with 500 ng of total RNA according to manufacturer's instructions. RNA complementary to the cDNA was removed using 2 units of *E. coli* RNase H and incubated at 37ºC for 20 min.

We fully amplified the coding regions of SWS1, LWS and RH1 visual opsin genes using universal primers designed to amplify visual opsin genes across snakes and squamates [16]. All fragments were amplified in 25 µl Polymerase Chain Reactions (PCR): 1X PCR buffer, 1.5 mmol (mM) of MgCl2, 50 µmol/l of deoxynucleotides, 0.4 µmol/l of each primer and 1 unit (U) Platinum Taq Polymerase and 100 ng of cDNA. PCR products were amplified by touchdown PCR with the following cycling parameters: initial denaturation at 95ºC for 5 min; 20 cycles of 1 min at 95ºC (denaturation), 30 s at 60ºC (annealing), and 1 min at 72ºC (extension) with a decrease of 0.5ºC per cycle; 15 cycles of 1 min at 95ºC (denaturation), 30 s at 50ºC (annealing), and 1 min at 72ºC (extension) followed by a final extension at 72ºC for 5 min. PCR products were run on a 1% agarose gel, excised in a Blue Light Transilluminator and purified with a PureLink Quick Gel Extraction Kit. PCR fragments were cloned with a StrataClone PCR Cloning Kit and corresponding chemically competent cells following the manufacturer's protocol. Transformed cells were grown overnight on agar medium treated with 100 mg/ml of Ampicillin and 1 ml of 2% X-GAL at 37ºC. Sixteen white colonies were picked (1 colony twirled in 50 µl of ultra-pure water) and used as DNA template in 25 µl PCR reactions: 1X PCR buffer, 1 mM of MgCl2, 80 µmol/l of deoxynucleotides, 0.2 µmol/l of M13F and M13R vector primers and 1U of BioTAQ Polymerase and 2 µl of DNA. The PCR had the following cycling parameters: initial denaturation at 95ºC for 10 min; 30 cycles of 15 s at 95ºC (denaturation), 30 s at 58ºC (annealing), and 1 min and 30 s at 72ºC (extension) and a final extension at 72ºC for 1.5 min. Eight positive clones were sequenced in both directions with M13 universal primers in an automated capillary Sanger sequencer.

To understand the evolution of short-wavelength vision in sea snakes we amplified and sequenced SWS1 exon 1 from genomic DNA. This offered a feasible alternative to fully sequencing SWS1 (> 8560 bp in the *Pseudonaja textilis* genome) from gDNA and the ethical and logistic problems of mRNA opsin sequencing, because exon 1 contains the sites (86, 90 and 93) that most substantially affect SWS1 pigment λ_{max} [5,18]. We therefore designed primers in an alignment of elapid SWS1 opsin genes, to amplify a fragment of exon 1 (between bp 136–346 in SWS1 exon 1 in *Aipysurus, Hydrophis, Laticauda, Notechis*) [SWS1F_Elapidae86 CCATCTTCATGGGCCTTGTCT; SWS1R_Elapidae86 CTGCCACTGTGCCTAGGAAG]. In most vertebrates, SWS1 pigment λ_{max} shifts are caused primarily by substitution at site 86, with F86 present in UV pigments (~360nm), S86/C86 (~416–422 nm, violet) and Y86 (~420–440nm, blue) in VS pigment [5,48–51]. Genomic DNA was extracted from tail scale clips with a DNeasy Blood&Tissue kit following the manufacturers' instructions. The exon 1 SWS1 opsin gene fragments were amplified in 25 µl Polymerase Chain Reactions (PCR): 5 µl PCR buffer, 0.4 µmol/l of each primer and 0.5 unit (U) of Immolase Taq Polymerase and 100 ng of cDNA. PCR products were amplified by touchdown PCR with the following cycling parameters: initial denaturation at 95ºC for 5 min; 20 cycles of 1 min at 95ºC (denaturation), 30 s at 60ºC (annealing), and 1 min at 72ºC (extension) with a decrease of 0.5ºC per cycle; 15 cycles of 1 min at 95ºC (denaturation), 30 s at 50ºC (annealing), and 1 min at 72ºC (extension) followed by a final extension at 72ºC for 3 min. PCR products were run on a 1% agarose gel and sequenced in both direction by an automated sequencer. Sequences were assembled in Geneious R10.

Microspectrophotometry

Snakes were dark adapted for at least two hours and euthanized with an overdose of barbiturate anaesthetic (Lethabarb). Eyes were removed under dim red light and the retina dissected under infra-red (IR) light with the aid of IR-image converters. Small pieces of retina were dissected and mounted in phosphate-buffered saline containing 8–10% (w/v) dextran (310–320 mOsmol kg^{-1}; pH 7.2) and compressed between two coverslips sealed with clear nail varnish. A single-beam, wavelength-scanning microspectrophotometer was used to measure the transverse absorbance (330–800 nm) of individual outer segments following methods previously used in snakes [41]. Absorbance spectra that satisfied established selection criteria [52,53] were retained for further analysis as described elsewhere [54]. An estimate of the wavelength of maximum absorbance (λ_{max}) of the visual pigment housed within each outer segment was made following the methods of [55,56]. For display, mean spectra for each photoreceptor class were overlain with an A_1 (rhodopsin) visual pigment template of the appropriate λ_{max} as proposed by [56].

Lens Spectral Transmission

Previous studies [4] suggest that the spectacle is largely light transmissive across all snake species. Lens spectral transmission was measured in adults of the following species: *Oxyuranus scutellatus* (3 specimens)*, Pseudonaja textilis* (x2)*, Notechis scutatus* (x2)*, Aipysusurus laevis* (x3)*, Hydrophis major* (x1), *H. peronii* (x2), *H. ocellatus* (x1) and *H. stokesii* (x1). A broad-band light source was beamed via an optical fiber through a UV transmissive cuvette containing the snake lens supported by a 3D printed billet. All the transmitted light was collected by an integrating sphere that was coupled to a spectrometer by an optical fiber.

QUANTIFICATION AND STATISTICAL ANALYSIS

Phylogenetic and Molecular Analysis

We investigated the selective strength acting on the opsin genes in Elapidae. We tested selection across the lineages (using branch models) and within the visual opsin codons (using site models). Codeml implemented in the PAML 4.7 package [44] was used to estimate nonsynonymous (dN) and synonymous (dS) substitution rates and the respective ratio (dN/dS, or ω) for the *sws1*, *lws* and *rh1* genes in terrestrial and sea snakes (Elapidae). Sequence alignment indels were removed if present in only one taxon or recoded as missing data if present in more. Branch models [19] ω to vary across branches in the tree and can be used to infer positive selection (ω > 1) acting in particular lineages (Figure S4). The simplest branch model (one-ratio) allows only one ω value across the tree, whereas the more complex free-ratio model allows independent ω for each branch. Branch models were used to estimate ω for two branch categories based on lineages and ecologies (Figure S4). Site models (M1a, M2a; M7β and M8 β&ω) allow ω to vary among sites [57]. Site models M2a and M8 β&ω were compared (using LRT) with the null hypothesis M1a and M7β, respectively. Bayes Empirical Bayes (BEB) [58] implemented in models M2a and M8 β&ω was used to identify sites inferred to be under positive selection for each visual opsin gene. Under branch-site models [58], ω can vary across both sites and lineages and this was used to determine whether positive selection at sites could be inferred for different lineages and ecologies (Figure S4). In each case, branch-site models were compared with the null hypothesis model M1a using LRT. Clade models (CmC) [19] assumes that some sites evolve conservatively across the phylogeny (two classes of sites where $0<\omega_0<1$ and $\omega_1=1$), whereas a class of sites is free to evolve differently among two or more partitions (e.g., $\omega_{D1}> 0$ and $\omega_{D1} \neq \omega_{D2} > 0$). CmC models were used across the same partitions (Figure S4) used for Branch and Branch-site models and compared with the null hypothesis M2a_rel [21] with a LRT.

Ancestral visual opsin gene sequences were estimated by marginal and joint reconstruction using codeml in PAML [44]. Additionally, we used parsimony in Mesquite [44] to reconstruct the ancestral state at SWS1 site 86.

To create a species tree, molecular data were obtained for 61 species across Elapidae (Elapiinae and Hydrophiinae). Sixty species were sampled in previous studies (genBank numbers in [1]), and one newly sampled taxon was collected in Bangladesh. This species was identified as *Hydrophis hendersoni* using morphological characters following [60], and standard protocols were used to generate a mitochondrial ND4 sequence from a preserved liver sample.

The final alignment comprised 3972 base pairs of mitochondrial genes cytochrome *b* (cytb), NADH dehydrogenase subunit 4 (ND4), and 16S small subunit ribosomal RNA (16S rRNA); in addition to nuclear coding genes recombination reactivating gene 1 (RAG-1) and oocyte maturation factor (c-mos). The MUSCLE plugin [61] with translation alignment of coding sequences in Geneious was used to generate the alignment, which was checked by eye.

Bayesian inference in BEAST v. 2.4.7 [46] was used to reconstruct a dated phylogeny. First, the Bayesian information criterion implemented in PartitionFinder2 [47] was used to determine the following best-fit partitioning scheme and substitution models: Mitochondrial coding codon positions 1 + 2: GTRig; mitochondrial coding codon positions 3: GTRg; nuclear coding codon positions 1 + 2: HKYig; nuclear coding codon positions 3: HKYg; 16S rRNA: GTRig. A secondary calibration taken from [1] was used to calibrate the root node age using a normal distribution with a mean of 30 Ma and 95% confidence intervals of 25 and 35 Ma. The Markov chain Monte Carlo was run for 20 000 000 generations with substitution parameters unlinked across partitions, a Yule tree model with a uniform distribution, and an uncorrelated and lognormally distributed relaxed clock model. Tree and clock models were linked across partitions. The chain was sampled every 20 000 generations, and convergence was assessed by examining likelihood plots and histograms, and

parameter effective sample sizes (ESS values) in Tracer v. 1.6. The first 600 sampled trees were excluded as burn-in, and a maximum credibility tree was generated from the remaining trees using Tree Annotator v. 2.4.6 [46].

Estimation of Polymorphism Balancing Selection

We estimated if balancing selection was acting on the SWS1 polymorphism by estimating absorption and fixation times for nuclear loci under neutral rates across all *Hydrophis*. We used the formula from Kimura and Ohta [62] to establish the fixation rate of polymorphism in *Hydrophis* under neutrality. The fixation rate is based on the assumption that polymorphism is maintained during speciation events in *Hydrophis* by strong selection pressure that prevented the loss of alleles through genetic drift and low turnover of selected alleles. Based on Kimura and Ohta [62] the average time to fixation is based on four times the effective population size (Ne). We used Ne estimated from the genomes of squamates and specifically in snakes [15] to estimate if the there was enough time for F86 and Y86 to fixate. We also estimated the absorption time for a neutral allele based on [29]. We estimated the elapsed time based on the number of generations during the *Hydrophis* radiation. We used *Hydrophis* crown group ages of 7.4 and 5 million years [1] and the generation time of 5 years [63].

Activity Patterns

Diel activity patterns were estimated using data on sea snake bycatch on commercial prawn fishing and scientific observer trawl vessels working in the Gulf of Carpentaria, Australia, between 1996 and 2001. All sea snakes collected on these vessels are accessioned in the Queensland Museum and have been reliably identified to species level. Trawl log data for each specimen include the date, trawl duration, trawl start and end time, and trawl start latitude/longitude and end latitude/longitude. To provide a proxy for activity rates in eight time bins (each of three hours duration), we calculated, for each species, the total number of snakes caught in each time bin divided by fishing effort (i.e. the total number of trawls in that time bin). Because the numbers of individuals caught varied among species, for each species in each time bin we divided the number of individuals caught by the total catch for that species.

Supplemental Items

Table S1. Identification and GenBank accession numbers of the samples used in this study. Related to STAR Methods.

Table S2. Known amino acid spectral tuning sites for all opsin gene and predicted peak absorbance (λ_{max}) for elapid snakes. Related to Figure 1.

Table S3. Ratio of synonymous to non-synonymous substitutions (dN/dS = ω) for elapid visual opsin gene sequences under branch-, site-, branch-site and clade models. Related to Figure 3.

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